

STIC-ILL

Vol 10

From: Holleran, Anne
Sent: Tuesday, January 27, 2004 2:06 PM
To: STIC-ILL
Subject: refs. for 08/070,099

479875

Please send copies of the following papers:

1. Srikumar Biomedicine (Chennai, India) (2002), 22(3&4): 94-104
2. Srikumar Current Science (Bangalore) (1998), 75(4): 387-391
3. Vaillant Cell and Tissue Research (1990) 260(1): 117-122

12904888

Anne Holleran
AU: 1642
Tel: 272-0833, after Jan 30
RM: Remsen, 3A14, after Jan 30

mailbox: 3A05, after Jan 30

Before Jan. 30:
tel: 308-8892
RM: 8E03
mailbox: 8E12

Cellular localization of intrinsic factor in pancreas and stomach of the dog

C. Vaillant, N.U. Horadagoda, and R.M. Batt

Departments of Veterinary Preclinical Sciences and Veterinary Pathology, University of Liverpool, Liverpool, United Kingdom

Accepted November 22, 1989

Summary. A cobalamin (vitamin B₁₂)-binding protein has recently been identified in canine pancreatic juice which is biochemically, immunochemically and functionally similar to canine gastric intrinsic factor. However, the cellular sources of both this pancreatic intrinsic factor and gastric intrinsic factor in the dog are not known. Antisera raised against canine gastric intrinsic factor have been used to examine the distribution of intrinsic factors in the canine pancreas and stomach. Immunoreactivity was demonstrated in duct cells but not acinar or endocrine cells in the pancreas, and in fundic peptic and pyloric gastric pit cells in stomach. All immunostaining was abolished by preabsorption of the antisera with purified canine gastric and pancreatic intrinsic factors. A cellular source of pancreatic intrinsic factor has not been previously described, and the demonstration of intrinsic factor-like immunoreactivity in two cell types in the canine stomach contrasts with its localization in a single cell type in the gastric mucosa of other mammalian species. Furthermore, immunoreactivity in pancreatic duct cells was detected at much higher dilutions of antisera than those required for staining of peptic and gastric pit cells. This suggests a higher concentration of antigen, and supports previous evidence that the pancreas is a major source of intrinsic factor in the dog.

Key words: Intrinsic factor – Pancreas – Pancreatic intrinsic factor – Stomach – Immunohistochemistry – Dog

Intrinsic factor (IF) is a cobalamin (vitamin B₁₂)-binding protein present in gastric juice which binds to specific receptors in the distal small intestine, and hence facilitates uptake of ingested cobalamin (Glass 1974; Mar-

coullis and Nicolas 1983). The other cobalamin-binding proteins in gastric juice, known as R binders, form unabsorbable complexes with cobalamin (Simons 1964; Grasbeck and Salonen 1976). Although IF is derived from gastric mucosa in all mammalian species studied (Glass 1974), immunohistochemical investigations have revealed species differences in the cellular sources of this glycoprotein (Jacob and Glass 1971; Levine et al. 1980, 1981, 1985).

The demonstration of impaired cobalamin absorption in human patients with exocrine pancreatic insufficiency (Cheney and Niemand 1932; Toskes et al. 1971) first implicated the pancreas in the normal handling of cobalamin, and introduced the possibility that the pancreas might be a source of IF. However, subsequent studies have suggested that pancreatic juice contributes to cobalamin absorption in man by degrading R binders, hence releasing cobalamin which can then bind to gastric IF (Allen et al. 1978; Marcoullis et al. 1980a; Toskes 1983; Marcoullis and Nicolas 1983).

In contrast to man, there is now considerable evidence that the pancreas plays a more fundamental role in the normal absorption of cobalamin in dogs. Firstly, despite the identification of IF in canine gastric juice (Marcoullis et al. 1980b) and of IF-cobalamin receptors in canine ileum (Marcoullis et al. 1980b; Seetharam et al. 1981; Levine et al. 1984), total gastrectomy does not appear to reduce cobalamin absorption (Abels and Muckerheide 1970; Abels et al. 1974, 1977). Secondly, low serum cobalamin concentrations have been reported in dogs with exocrine pancreatic insufficiency (Batt and Morgan 1982) and ligation of pancreatic ducts results in a severe malabsorption of cobalamin (Abels et al. 1974, 1977; Simpson et al. 1989) which can be reversed by oral administration of canine pancreatic juice (Simpson et al. 1989) or extracts of canine pancreas, even in dogs with total gastrectomy (Abels et al. 1974, 1977). Thirdly, a cobalamin-binding protein which is biochemically and immunochemically similar to canine gastric IF has recently been identified in canine pancreatic juice,

Send offprint requests to: Dr. C. Vaillant, Department of Veterinary Preclinical Sciences, The University, Liverpool L69 3BX, UK

nd has been shown to promote ileal absorption of cobalamin by a physiological route (Batt et al. 1989; Batt and Horadagoda 1989). However, the cellular origins both of this pancreatic IF and also of gastric IF in the dog are not known.

Antisera raised against human and porcine IF have been used to localize IF in the gastric mucosa of various mammals (Levine et al. 1980, 1981, 1985), but canine IF appears to be immunohistochemically distinct from other mammalian IFs and shows weak or no cross-reactivity with such antisera (Yamaguchi et al. 1969; Abels and Luckerheide 1969, 1970). In the present study, antisera used against IF purified from canine gastric mucosa (Batt et al. 1989) have been used to localize IF-immunoreactivity in canine pancreas and stomach. Preliminary parts of these findings have been published (Vaillant et al. 1986; Horadagoda et al. 1986).

Materials and methods

Gastric and pancreatic IFs were purified by affinity chromatography, as previously described (Horadagoda and Batt 1985; Batt et al. 1989). Successful purification of IFs and separation from binders was demonstrated by showing a lack of cobinamide inhibition of cobalamin-binding capacity, and a single band for both gastric and pancreatic IFs on SDS-polyacrylamide gel electrophoresis, corresponding to a molecular mass of approximately 53 kDa (Batt et al. 1989). The production of rabbit polyclonal antisera (R3 and R4) to purified canine gastric IF has been reported (Batt et al. 1989).

Tissue samples were taken from stomach (corpus and pyloric regions), pancreas (splenic and duodenal lobes, main duct) and salivary glands (parotid, mandibular) of normal dogs, from the pancreas of dogs with naturally occurring exocrine pancreatic insufficiency due to acinar atrophy (Hill et al. 1971), and from the stomach and pancreas of normal cats. Tissues were fixed in either fixative fluid or 4% formaldehyde solution and processed into paraffin blocks. Samples of normal human stomach, processed in the same way, were available from a previous study (Taylor et al. 1981). Sections of formalin-fixed human pancreatic tumors containing normal pancreas were kindly donated by Dr. G. Rothery, Department of Pathology, University of Liverpool Medical School. The distribution of gastric IF-like immunoreactivity was examined by use of the peroxidase anti-peroxidase (PAP) method (Sternberger et al. 1970). Sections were incubated for 24 h with antisera R3 and R4 at serial dilutions from 1:100 to 1:64000, then for each with goat anti-rabbit IgG and rabbit PAP, both at 1:50 dilutions. The latter two antisera were purchased from ICN Biologics, Inc., UK. Sites of antibody binding were revealed by incubating sections for 10 min in substrate consisting of 0.025% diaminobenzidine and 0.015% hydrogen peroxide in 0.05 mol/l Tris buffer, pH 7.6. All reagents were purchased from Sigma, UK. In order to reduce background staining, endogenous peroxidase was blocked by incubating sections in aqueous 1% hydrogen peroxide for 20 min prior to staining, and by rinsing sections after each incubation in phosphate buffer (0.1 mol/l, pH 7.2) containing 2.5% v/v chloroform (Grube 1980). Crossreactivity of the two anti-gastric IF antisera with pancreatic IF was established by Western blotting (Batt et al. 1989). Specificity of the immunostaining was investigated by prior incubation of the primary antisera with purified gastric or pancreatic IFs (1 nmol IF/ml antiserum at 1:1000 dilution, for 48 h), and by reacting primary antisera with normal rabbit serum (1:50). The absorptions of purified IFs used for these absorptions were those previously described (Batt et al. 1989).

Results

Immunohistochemical localizations of IF-like immunoreactivity in canine stomach, pancreas and salivary gland, were identical with R3 and R4 antisera, and all staining was abolished when the antisera were preabsorbed with either purified gastric IF or purified pancreatic IF. No staining was obtained when normal rabbit serum was substituted for the antisera.

In the pancreas, immunoreactivity was localized in the epithelium lining exocrine ducts: no staining was observed in pancreatic acinar or endocrine cells. Immunoreactivity was present in all epithelial cells in the main pancreatic duct and in interlobular ducts within the body of the pancreas, and occasionally also in the lumen (Fig. 1). In smaller intralobular ducts, there was an increasing proportion of non-immunoreactive cells as the ducts decreased in size (Fig. 2). A similar pattern of immunoreactivity was seen in the pancreas of dogs with exocrine pancreatic insufficiency in which there was a severe loss of exocrine cells but retention of the duct system and endocrine cells (Fig. 3). Immunoreactivity in the pancreas was detected by antisera R3 and R4 diluted to 1:64000.

In contrast, the optimal dilution of both antisera for demonstrating IF-like immunoreactivity in the stomach was 1:1000. Staining was observed in peptic cells in the fundic mucosa (Fig. 4a, b), and in columnar epithelial cells lining in the lower half of gastric pits in the pyloric mucosa (Fig. 5a, b). In the latter region, staining gradually decreased in epithelial cells closer to the surface of the mucosa, whereas there was an abrupt loss of immunoreactivity at the base of the gastric pits at junctions with pyloric glands (Fig. 5a). In the gastric pit cells, immunoreactivity was confined to the cytoplasm below the apical mucinogen granules (Fig. 5b).

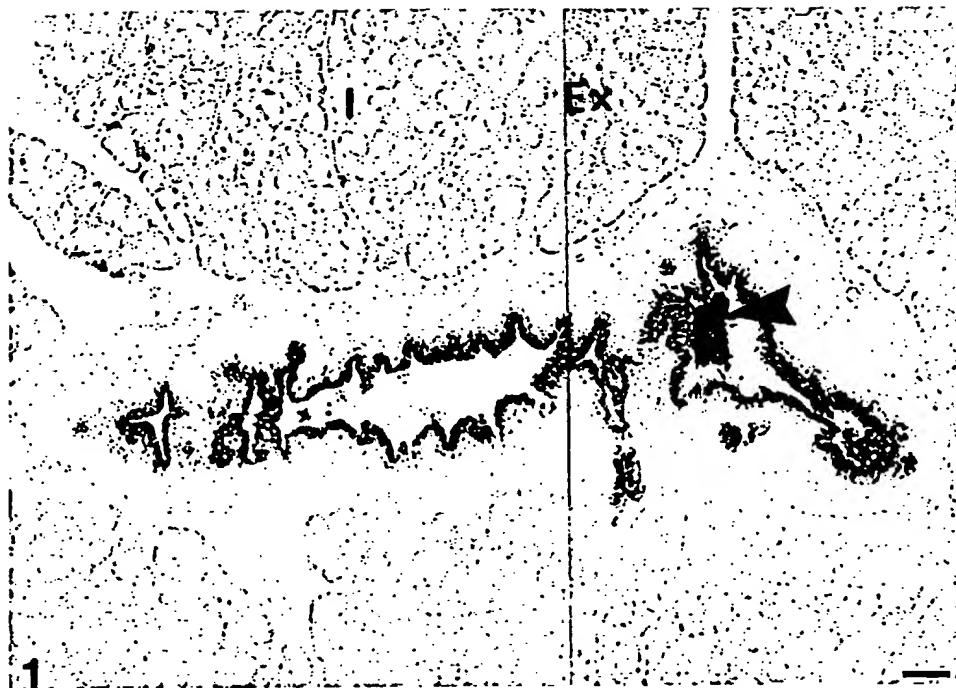
In canine salivary glands, immunoreactivity was localized in only a few isolated epithelial cells in striated ducts in the mandibular gland (Fig. 6a, b), and none was observed in the parotid gland. Localizations were not obtained at dilutions of antisera higher than 1:1000.

No localizations were obtained in human and feline pancreas and stomach with antiserum R3. On the other hand, antiserum R4 did give weak localizations in human parietal cells only, and this staining was abolished by preabsorption with canine IF.

Discussion

Recent investigations have demonstrated that the pancreas is an important source of IF in the dog (Batt et al. 1989; Batt and Horadagoda 1989). The present study has pursued these findings and represents the first demonstration of the cellular source of IF in canine pancreas.

The localization of IF-like immunoreactivity in the pancreas and also in two cell types in the gastric mucosa in dogs contrasts with findings in other mammalian species where immunoreactivity appears to be confined to one cell type in either the fundic or pyloric regions of



Figs. 1-6. Canine tissues, immunoperoxidase localization of IF-like immunoreactivity and counterstaining with haematoxylin

Fig. 1. Canine gastric IF-like immunoreactivity is demonstrated in all lining cells and in the lumen (arrow) of an interlobular duct: exocrine acini (Ex) and islets (I) are unstained. $\times 120$. Bar: 50 μm

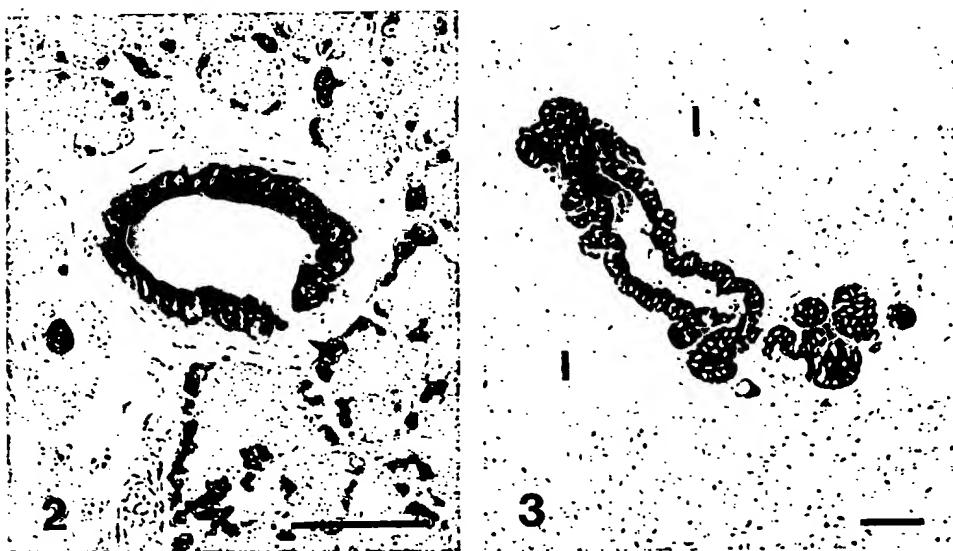


Fig. 2. IF-like immunoreactivity is present in the majority of cells lining an intralobular duct in dog pancreas except where a smaller duct branches off (arrow). $\times 360$. Bar: 50 μm

Fig. 3. Pancreas from a dog with pancreatic acinar atrophy. Acinar tissue is severely reduced; IF-like immunoreactivity is present in ducts but not in surrounding islet tissue (I). $\times 190$. Bar: 50 μm

the stomach (Jacob and Glass 1971; Levine et al. 1980, 1981, 1985). IF-immunoreactivity has been localized in parietal cells in man, guinea pigs and rabbits (Jacob and Glass 1971; Levine et al. 1980, 1981, 1985), but occurs in peptic cells in rats (Levine et al. 1981). The pig is quite different since IF-immunoreactivity has been demonstrated in cells lining the lower half of gastric pits in the pyloric antrum in this species (Levine et al. 1981). Thus, the localization of IF-like immunoreactivity in peptic cells and pyloric gastric pit cells in canine stomach indicates similarities both with the rat and with the pig, respectively. Antisera against canine gastric IF did not stain human and feline stomach or pancreas, apart from very weak staining of human parietal cells with antiserum R4, confirming earlier reports of immunochemical

differences between IFs from dog and other mammalian species (Yamaguchi et al. 1969; Abels and Muckerheide 1969, 1970).

The possibility that a pancreatic IF might exist has been considered but has been refuted, at least in man (Allen et al. 1978; Marcoullis et al. 1980a; Marcoullis and Nicolas 1983; Toskes 1983; Carmel et al. 1983). However, pancreatic IF has been identified in the dog, and this protein has been shown to resemble canine gastric IF biochemically, immunochemically and functionally (Batt et al. 1989; Batt and Horadagoda 1989). The present study has extended these observations, and has demonstrated that pancreatic duct epithelium is the cellular source of this IF in canine pancreatic juice. The output of IF by the canine pancreas has been examined

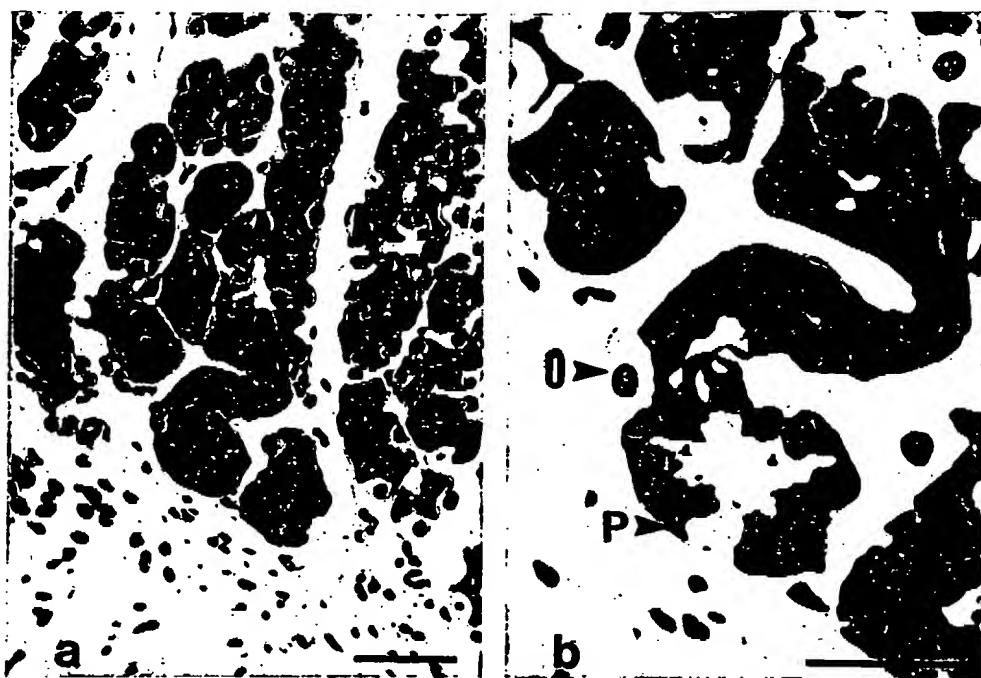


Fig. 4a, b. IF-like immunoreactivity in peptic cells (P), but not oxytic cells (O) in canine fundic glands. a $\times 280$, b $\times 720$. Bars: a 50 μm ; b 25 μm

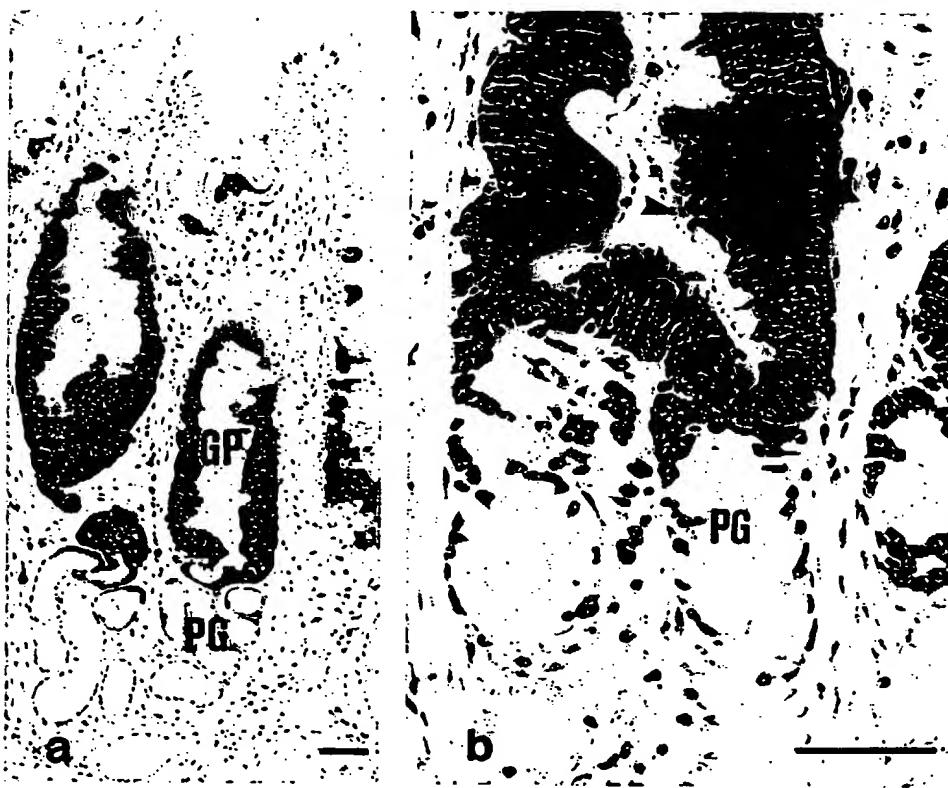


Fig. 5a, b. Immunoperoxidase demonstration of canine gastric IF-immunoreactivity in epithelial cells lining gastric pits in canine pyloric antral mucosa. Immunoreactivity increases towards the base of the gastric pits (GP), but is absent from the pyloric glands (PG). In individual gastric pit cells, staining occurs in the cytoplasm (large arrow) below the apical mucinogen granules (small arrow). a $\times 120$, b $\times 360$. Bars: 50 μm

following intravenous administration of secretin and cholecystokinin (CCK) (Batt et al. 1989). Stimulation of fluid output by secretin was associated with a relatively transient peak of IF, suggesting washout of material present in the lumen of ducts, whereas CCK resulted in a significantly greater output of IF which paralleled outputs of trypsin and protein, consistent with stimulation of IF secretion. Together with the identification of

the cellular origin of pancreatic IF, these findings suggest that CCK can stimulate protein secretion by duct cells by a direct or indirect mechanism.

The demonstration of IF-like immunoreactivity in pancreatic ducts in dogs with pancreatic acinar atrophy suggests that these animals may retain the ability to produce pancreatic IF. Nevertheless, low serum concentrations of cobalamin (Batt and Morgan 1982) could reflect

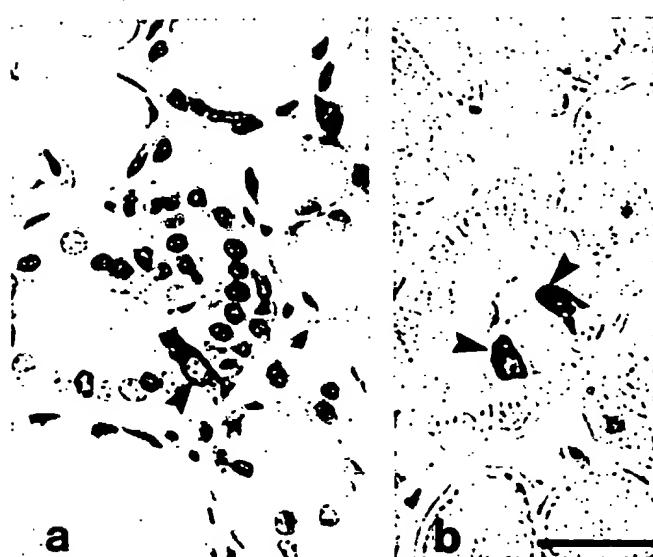


Fig. 6a, b. IF-like immunoreactivity occurs in isolated cells (arrows) in small striated ducts in mandibular gland (b phase contrast). $\times 550$. Bar: 25 μm

impaired absorption of the vitamin perhaps due to reduced pancreatic fluid secretion (Sateri 1975) and hence defective output of pancreatic IF. However, alternative possibilities include reduced degradation of R-binders by pancreatic enzymes, or overgrowth of intestinal bacteria, and these have been discussed elsewhere (Simpson et al. 1989).

Kudo et al. (1987) have reported R binder-like immunoreactivity in ducts of human pancreas, a distribution which resembles that described here for IF in canine pancreas. The cellular source of canine R binders present in pancreatic juice (Batt et al. 1989) is unknown but it is possible that these may be derived from the same cell as canine pancreatic IF. In stomach and salivary gland, no other similarities were seen between our cellular localizations of IF-like immunoreactivity in dog and that of R binder-like immunoreactivity in man (Kudo et al. 1987).

Antigenicity was detected in the pancreas at much higher dilutions of antisera than those needed for staining in the stomach. The antisera were both raised against gastric IF and, although there are immunochemical similarities between canine pancreatic and gastric IFs (Batt et al. 1989), it is possible that the antisera may have a higher affinity for pancreatic than for gastric IF following tissue fixation. However, it is more likely that the difference in staining sensitivity reflects a much higher concentration of antigen in pancreatic duct cells than in peptic and gastric pit cells. This suggestion is supported by the five- to ten-fold higher output of IF in pancreatic secretion stimulated by secretin and CCK, respectively, compared with pentagastrin-stimulated IF output in gastric juice (Batt et al. 1989). While measurements of tissue concentrations of IF are needed to substantiate this suggestion, it is consistent with other evidence for a major role of the pancreas in the normal

absorption of cobalamin by dogs (Batt et al. 1989; Batt and Horadagoda 1989; Simpson et al. 1989).

Acknowledgements. NUH was supported by the Association of Commonwealth Universities and the study was supported in part by a Scientific Investigation Grant from The Royal Society.

References

- Abels J, Muckerheide MM (1969) Antigenic species specificity of the gastric intrinsic factor. Proc Cent Soc Clin Res 42nd Annual Meeting: 843
- Abels J, Muckerheide MM (1970) Absorption of vitamin B_{12} in dogs. Clin Res 18:530
- Abels J, Muckerheide MM, van Kapel J, Lindemans J (1974) A dual role for the dog pancreas in the absorption of vitamin B_{12} . Program, Am Soc Hematol Meeting: 95
- Abels J, van Kapel J, Lindemans J (1977) A dual function of the dog pancreas in absorption of vitamin B_{12} by secretion of a prointrinsic factor and trypsinogen. Neth J Med 20:26
- Allen RH, Seetharam B, Allen NC, Podell E, Alpers DH (1978) Correction of cobalamin malabsorption in pancreatic insufficiency with a cobalamin analogue that binds with high affinity to R protein but not to intrinsic factor. In vivo evidence that a failure to partially degrade R protein is responsible for cobalamin malabsorption in pancreatic insufficiency. J Clin Invest 61:1628-1634
- Batt RM, Horadagoda NU (1989) Gastric and pancreatic intrinsic factor-mediated absorption of cobalamin in the dog. Am J Physiol 257:R9344-R9349
- Batt RM, Horadagoda NU, McLean L, Morton DB, Simpson K (1989) Identification and characterization of a pancreatic intrinsic factor in the dog. Am J Physiol 256:G517-G523
- Batt RM, Morgan JO (1982) Role of serum folate and vitamin B_{12} concentrations in the differentiation of small intestinal abnormalities in the dog. Res Vet Sci 32:17-22
- Carmel R, Abramson SB, Renner IG (1983) Characterization of pure human pancreatic juice: cobalamin content, cobalamin-binding proteins and activity against human R binders of various secretions. Clin Sci 64:193-205
- Cheney G, Niemand F (1932) A possible relationship of pancreatic insufficiency to Addison-Biermer (pernicious) anaemia. Arch Int Med 49:925-933
- Glass GB (1974) Gastric Intrinsic Factor and Other Vitamin B_{12} Binders: Biochemistry, Physiology, Pathology and Relation to Vitamin B_{12} Metabolism. Georg Thieme Publishers, Stuttgart
- Grasbeck R, Salonen E-M (1976) Vitamin B_{12} . Prog Fd Nutr Sci 2: 193-231
- Grube D (1980) Immunoreactivities of gastrin (G-) cells. 11 Non-specific binding of immunoglobulins to G-cells by ionic interactions. Histochemistry 66:149-167
- Hill FWG, Osborne AD, Kidder DE (1971) Pancreatic degenerative atrophy in dogs. J Comp Pathol 81:321-330
- Horadagoda NU, Batt RM (1985) Purification of canine intrinsic factor by affinity chromatography. Biochem Soc Trans 14:133-134
- Horadagoda NU, Batt RM, Vaillant C, Morton DB, Simpson KW (1986) Identification and characterization of a pancreatic intrinsic factor in the dog. Gastroenterology 90:1464
- Jacob E, Glass GB (1971) Localization of intrinsic factor and complement fixing intrinsic factor-intrinsic factor antibody complex in parietal cell of man. Clin Exp Immunol 8:517-527
- Kudo H, Inada M, Ohshio G, Wakatsuki Y, Ogawa K, Hamashima Y, Miyake (1987) Immunohistochemical localization of vitamin B_{12} R binder in the human digestive tract. Gut 28:339-345
- Levine JS, Nakane PK, Allen RH (1980) Immunocytochemical localization of human intrinsic factor: the nonstimulated stomach. Gastroenterology 79:493-502

Levine JS, Nakane PK, Allen RH (1981) Phylogenetic differences in the site of synthesis of intrinsic factor (IF): an immuno-electronmicroscopic study. *Gastroenterology* 80:1210

Levine JS, Allen RH, Alpers DH, Seetharam B (1984) Immunocytochemical localization of the intrinsic factor-cobalamin receptor in dog ileum: distribution of intracellular receptor during cell maturation. *J Cell Biol* 98:1111-1118

Levine JS, Magree L, Allen RH, Lusk LB (1985) Ultrastructural immunocytochemical localization of intrinsic factor in isolated rabbit gastric glands. *Gastroenterology* 88:1474

Marcoullis G, Nicolas J-P (1983) The interactions of cobalamin in the gastrointestinal tract. In: Glass GBJ, Sherlock P (eds) *Progress in Gastroenterology*, vol. IV. Grune and Stratton, New York, pp 133-172

Marcoullis G, Parmentier Y, Nicolas J-P, Jimenez M, Gerard P (1980a) Cobalamin malabsorption due to nondegradation of R proteins in the human intestine: inhibited cobalamin absorption in exocrine pancreatic dysfunction. *J Clin Invest* 66:430-440

Marcoullis G, Rothenberg SP, Labombardi VJ (1980b) Preparation and characterization of proteins in the alimentary tract of the dog which bind cobalamin and intrinsic factor. *J Biol Chem* 255:1824-1829

Sateri H (1975) Investigations on the exocrine pancreatic function in dogs suffering from chronic exocrine pancreatic insufficiency. *Acta Vet Scand Suppl* 53:1-86

Seetharam B, Alpers DH, Allen RH (1981) Isolation and characterization of the ileal receptor for intrinsic factor-cobalamin. *J Biol Chem* 256:3785-3790

Simons K (1964) Vitamin B₁₂ binders in human body fluid and blood cells. *Soc Sci Fennica Com Biol* 27:3-94

Simpson KW, Morton DB, Batt RM (1989) Effect of exocrine pancreatic insufficiency on cobalamin absorption in dogs. *Am J Vet Res* 50:1233-1236

Sternberger LA, Hardy PH Jr, Cuculis JJ, Meyer HG (1970) The unlabelled antibody enzyme method of immunohistochemistry. Preparation and properties of soluble antigen-antibody complex (horseradish peroxidase-antihorseradish peroxidase) and its use in identification of spirochetes. *J Histochem Cytochem* 18:315-333

Taylor IL, Calam J, Rotter JI, Vaillant C, Samloff IM, Cook A, Simkin E, Dockray GJ (1981) Family studies of hypergastrinemic, hyperpepsinogenemic I duodenal ulcer. *Ann Intern Med* 95:421-425

Toskes PP (1983) Intestinal absorption defects of cobalamin (vitamin B₁₂) and their differential diagnosis in gastrointestinal disease. In: Glass GBJ, Sherlock P (eds) *Progress in Gastroenterology*, vol. IV. Grune and Stratton, New York, pp 173-188

Toskes PP, Hansell J, Cerda J, Deren JJ (1971) Vitamin B₁₂ malabsorption in chronic pancreatic insufficiency. Studies suggesting the presence of a pancreatic "intrinsic factor". *N Engl J Med* 284:627-632

Vaillant C, Horadagoda NU, Batt RM (1986) The pancreas is a major source of intrinsic factor in the dog. *Can J Physiol Pharmacol* July [Suppl]:154-155

Yamaguchi N, Weisberg H, Glass GBJ (1969) Intestinal vitamin B₁₂ absorption in the dog. *Gastroenterology* 56:925-935

Co
of
in

Mat

¹ Dep² Dep

Accet

Sum
minc
cone
ing
inter
by li
cally
term
"spi
quar
depe
of th
inter
mine
speci
good
lengl
HCS
idast
pedi
to b
cord
feed
from
(cent
spini
HCSKey
visio
hus (Subt
a vi
(DjaSend
Zellbi
FRG